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TITLE: Acute Pancreatitis as a Model to Predict Transition of Systemic Inflammation to Organ Failure in Trauma and Critical Illiness

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CONTRACTING ORGANIZATION: UNIVERSITY OF PITTSBURGH, THE
PITTSBURGH, PA 15213-3320

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14. ABSTRACT
Trauma, extensive burns, bacterial infections, and acute pancreatitis (AP) are common conditions of tissue injury and immune system activation that can result in the systemic inflammatory response syndrome (SIRS). Surprisingly, about half of the patients with SIRS quickly recover, while the others develop a multiorgan dysfunction syndrome (MODS). SIRS and MODS do not occur immediately: SIRS evolves over a 4-12 hour period, while MODS evolves over 12-24 hours. Vascular leak syndrome (VLS) is a critical component of the transition from SIRS to MODS. Understanding the mechanism by which SIRS triggers VLS and progresses to MODS is critical to correctly model disease course thereby aiding in treatment of patients. In this report, we tested the effect of severe acute pancreatitis patient serum on the viability of human vascular endothelial cells grown in a monolayer. The experiments show the disruption of the endothelial monolayer as an in vitro model of VLS and allow a quantitative measure by evaluating % cell viability. The results demonstrate differences in viability between AP patients and normal healthy volunteers.

15. SUBJECT TERMS
Pancreatitis, systemic, inflammation, vascular leak, multiple organ dysfunction, biomarkers, endothelium, viability

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The problem being addressed is the unknown mechanism(s) in patients with acute pancreatitis, multiple trauma, severe burn, or sepsis responsible for the unpredictable progression of systemic inflammation to the vascular leak syndrome (VLS), which in turn leads to multi-organ dysfunction syndrome (MODS). Our experimental approach is designed to understand and predict progression from systemic inflammation to MODS. The primary observation is that serum or plasma from patients with severe acute pancreatitis (AP) or trauma with VLS is toxic to endothelial cells.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Pancreatitis, systemic, inflammation, vascular leak, multiple organ dysfunction, biomarkers, endothelium, viability

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Aim 1. Define the clinical setting in which SIRS progresses, and fails to progress, to VLS and MODS using molecular and clinical measures. (months 4-36)

Aim 2. Determine the effect of serum from patients with SIRS ±VLS as well as Ang-2 and other target molecules (identified in Aim 3) on human organ-derived endothelial cells in terms of morphology, gene activation, and mode of cell death. (months 4-36)

Aim 3. Identify serum molecule(s) that best predict specific in vitro changes in endothelial cells (Aim 2) as well as which molecule(s) and endothelial cell changes best predict clinical progression to MODS (Aim 1). (months 6-36)

What was accomplished under these goals?

The specific objectives under **Aim 1** was to get Institutional Review Board (IRB) approval so patients can be recruited into the study. University IRB approval was received February 4, 2015 with the category of Less Than Minimal Risk study. This was followed by the US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protections Office (HRPO) approval on February 28, 2015. The study is approved for the enrollment of 61 subjects. Additionally, a research nurse coordinator, Weiping DeBlasio, RN, was hired July 1, 2015 to coordinate patient recruitment, consents, and sample collection. There were 3 subjects enrolled in the study year 1.

The specific objectives under **Aim 2** was to first isolate and characterize human organ-specific endothelial cells for use in the project. This will be an important ongoing process throughout the project in order to meet the goals of this study. Human intestinal vascular endothelial cells were isolated from waste surgical specimens collected directly from the operating room in order to keep the tissue viable (October 2014 – September 2015). The cells were isolated utilizing mechanical and enzymatic processes allowing early clusters of endothelial cells to establish on fibronectin coated tissue culture dishes. Following

"weeding" of the cultures over the initial 2 weeks in vitro, the clusters of endothelial cells are purified using a CD31 magnetic bead selection and these cells are then put back into routine culture. Purified vascular endothelial cultures from intestine display a classic cobblestone morphology and will display avid uptake of acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-ac-LDL), all pathognomoic features of their endothelial heritage. For all experiments, monolayers of vascular endothelial cells are allowed to achieve confluence for a 5 day time period prior to experimentation. Prior experimentation has demonstrated that this is an appropriate time point to allow for optimal expression of organ specific patterns of cell adhesion molecules, etc. Early passage purified cells are cryogenically stored for later experiments. The viability, morphology, and physiology of newly isolated cells were assessed using the following assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) where MTT is converted to formazan in healthy cells via mitochondrial enzymes, and lactate dehydrogenase (LDH) release assay that measures the release of intracellular LDH from damaged plasma membrane (viability); F-actin stress fiber staining with phalloidin after treatment with or without activators (LPS); and autophagy, a conserved cellular process that mediates degradation of intracellular components including proteins and organelles via LC3B containing autophagosome formation and degradation in the autolysosome, measured with LCB3 immunostaining. The figures below show that the isolated vascular endothelial cells behave as expected.

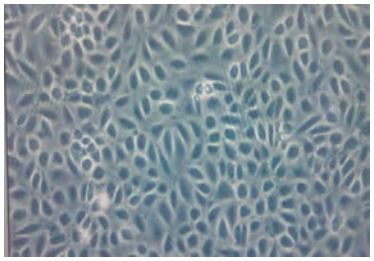


Figure 1. Phase contrast image of confluent human intestinal vascular endothelial cells. 20X magnification

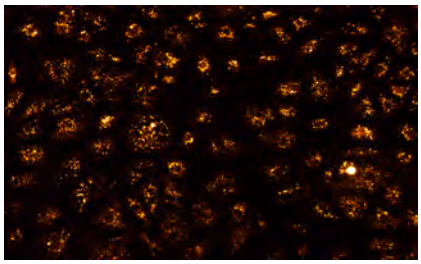


Figure 2. Normal Dil-Ac-LDL uptake in human intestinal vascular endothelial cells. 20X magnification

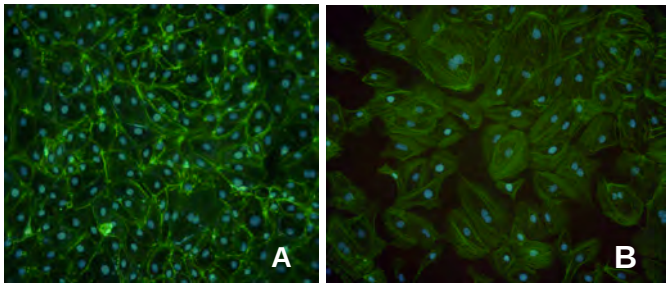


Figure 3. F-actin fibers stained with phalloidin (green) and nuclei stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) in human intestinal vascular endothelial cells in normal growth medium (A) and treated with 100ng/ml LPS for 6 h (B). Images demonstrate thickening of stress fibers due to activation of endothelial cells with LPS treatment. 20X magnification

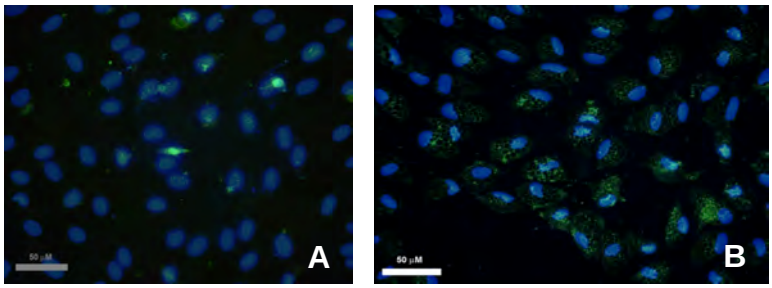


Figure 4. Autophagy is shown to increase in human intestinal vascular endothelial cells when they are serum starved for 24 h (B) in comparison to cells grown in normal growth medium (A). LC3B on autophagosomal membranes is stained green. Nuclei are stained blue with DAPI.

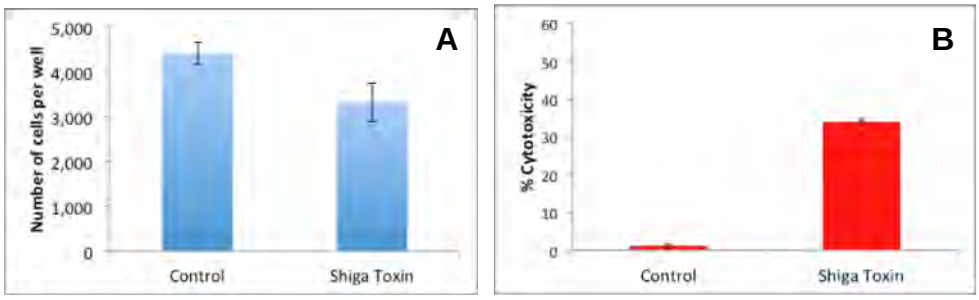


Figure 5. The MTT and LDH release assays demonstrate the viability of human intestinal vascular endothelial cells. In this experiment, 2,500 cells were seeded in each fibronectin-coated well. Controls were treated with normal cell growth medium and experimental wells were treated with normal cell growth medium containing a known endothelial cell toxin shiga toxin-1 (250 pg/ml). Cells were incubated for 48 h. The shiga toxin clearly inhibits cell proliferation (A) as shown by the MTT results and is toxic to the cells as indicated by the increase in LDH release into the cell culture medium (B). This data shows that the endothelial cells respond as they should in the given conditions.

Prior to recruitment of patients into this study, serum samples collected from patients diagnosed with acute pancreatitis (mild, moderate or severe) from an approved University of Pittsburgh IRB (Pancreatitis-associated Risk of Organ Failure or PROOF) were screened for toxicity to the HIMEC using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay where MTT is converted to formazan in healthy cells via mitochondrial enzymes. The HIMEC were treated with 10% of the serum sample added to the basal medium (MCDB131) for 24 and 48-hour time points. Low- and mid- abundance proteins in the serum samples that reduced cell survival to 40% or less were then enriched using Bio-Rad ProteoMiner Protein Enrichment Kit. The effect of the enriched fraction containing the low- and mid-abundance proteins was then compared to the fraction containing the high abundance proteins on the endothelial cells. Total protein in each fraction was determined using the BCA protein assay (Thermo Scientific Pierce) so equal amounts of total protein could be added to the cells. The HIMEC were treated with 3% of the fractions added to the basal medium for 48 hours. The results show that the enriched fraction containing the low- and mid- abundance proteins were toxic to the endothelial cells. Cell viability was also tested using propidium iodide. Propidium iodide intercalates DNA inside of cells with damaged cell membranes (does not enter viable cells). The endothelial cells were treated with 5%, 10%, and 20% of the serum samples added to the complete growth medium (MCDB131) for 24 and 48-hour time points. As can be seen in the figures, serum from patients with a severe acute pancreatitis category does not always have the greatest toxic effect on the vascular endothelial cells using this method.

Table 1 - Severity categories following the Revision of Atlanta Classification[3].

Acute pancreatitis severity	Organ failure and local or systemic complications
Mild acute pancreatitis	- No organ failure - No local or systemic complications
Moderately severe acute pancreatitis	- Transient organ failure (resolves in 48 hours)
	Local or systemic complications without persistent organ failure
Severe acute pancreatitis	- Persistent organ failure (single or multiple)

Table 2. Viability of Human Intestinal Vascular Endothelial Cells Treated with Acute Pancreatitis Patient Serum as Measured by MTT Assay

Sample #	***ap.severity.(RAQ)***	***pain.day.proof***	***% Survival EC 24 h***	***% Survival EC 48 h***	***% Survival Low & Mid Abund***	***% Survival High Abund***
PR0005	severe	5	61	48		
PR0009	severe	5	76	72		
PR0019	severe	5	58	37		
PR0025	moderate	5	44	53		
PR0031	moderate	5	59	68		
PR0035	moderate	4	58	64		
PR0038	severe	4	64	84		
PR0051	severe	5	59	91		
PR0061	severe	4	52	52		
PR0064	severe	5	65	70		
PR0077	moderate	4	38	38	3	59
PR0078	severe	5	69	90		
PR0086	severe	5	71	81		
PR0088	severe	4	87	78		
PR0094	severe	5	43	43	3	79
PR0096	severe	4	81	94		
PR0103	severe	5	71	78		
PR0104	severe	5	71	87		
PR0105	severe	5	54	49		
PR0106	severe	4	73	97		
PR0107	severe	5	70	83		
PR0109	moderate	4	65	60		
PR0110	moderate	4	36	47		
PR0113	moderate	5	49	51		
PR0128	moderate	4	55	52		
PR0133	severe	3	50	37	4	99
PR0137	severe	4	48	38	4	117
PR0138	moderate	4	75	61		
PR0140	moderate	5	74	61		
PR0146	moderate	5	62	55		
PR0153	moderate	4	51	60		
PR0155	moderate	4	71	79		
PR0163	moderate	3	40	49		
PR0165	moderate	4	76	103		
PR0173	moderate	3	106	149		
PR0174	severe	4	53	45		
PR0178	moderate	3	78	70		
PR0178	moderate	5	84	92		
PR0188	moderate	5	94	148		
PR0197	moderate	5	53	46		

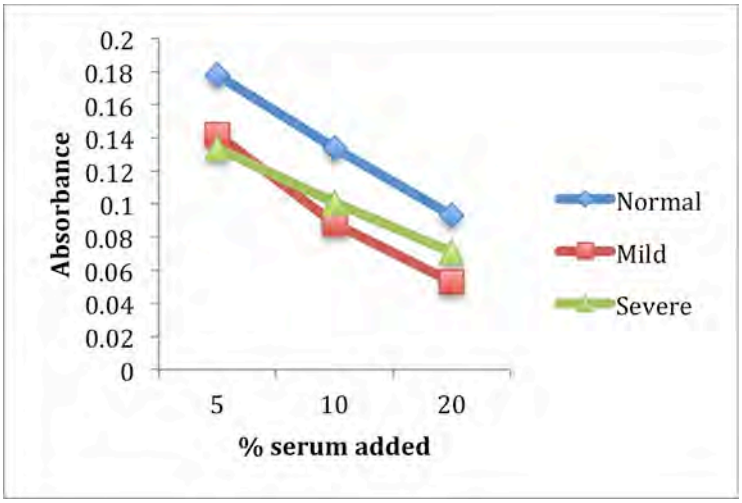


Figure 6. Viability of human vascular endothelial cells treated with normal subject, mild and severe acute pancreatitis patient serum 48 h (MTT assay).

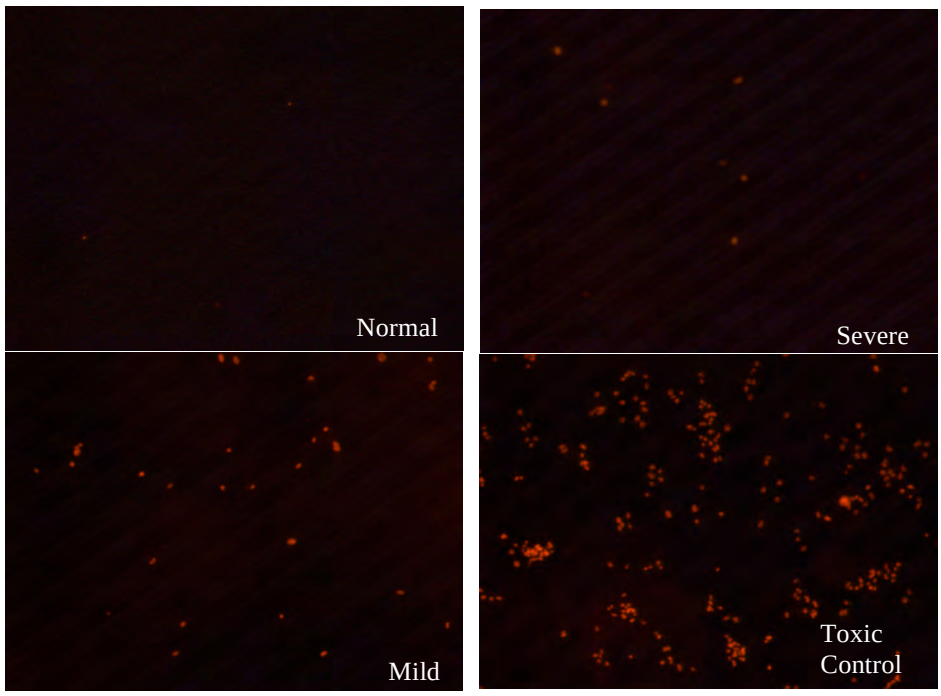
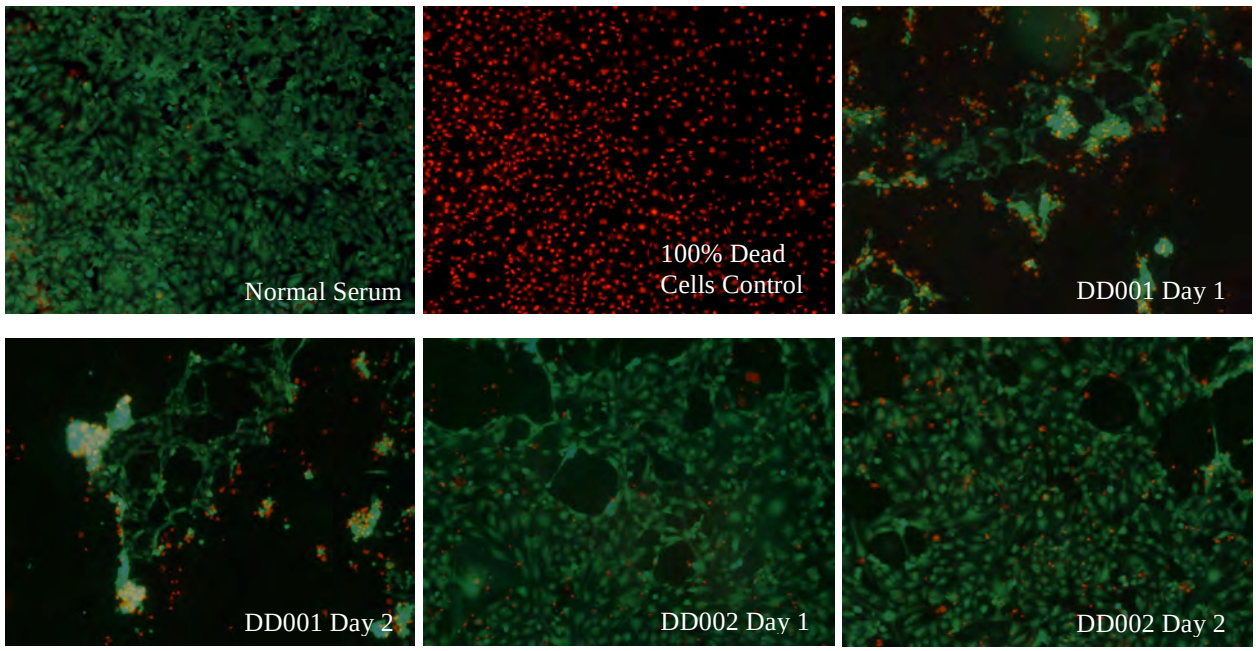
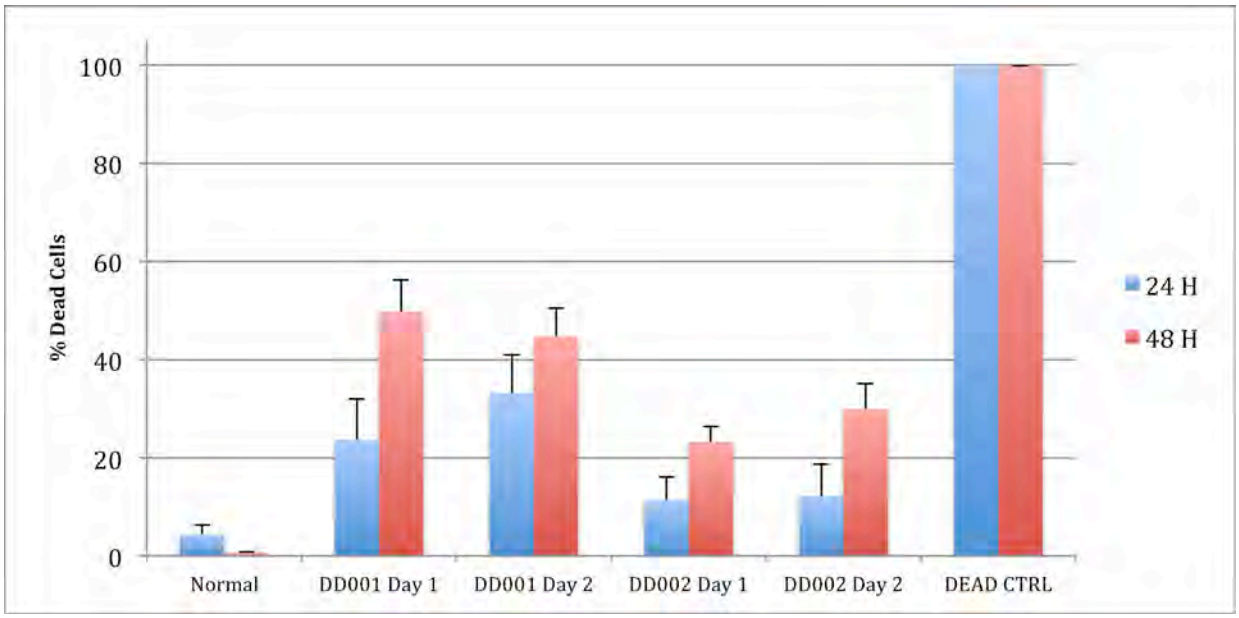


Figure 7. Viability of human vascular endothelial cells treated with normal subject, mild and severe acute pancreatitis patient serum 48 h (Propidium iodide assay). 20X magnification.

Three subjects were enrolled into our study during the last quarter of the first year. Two males and one female diagnosed with severe pancreatitis. The two males were of age 67 (DD001) and 39 years old (DD003). The female subject was 52 years old (DD002). Blood was collected on days 1, 2, 3, 4, 5, 6, and 7 for patients 1 and 3. Blood was collected on days 1, 2, 3, and 4 for patient 2 who was discharged on day 4. Serum from days 1 and 2 of patients DD001 and DD002 were studied in this experiment. These two subjects were selected because of the differences of severity of acute pancreatitis between the two patients. DD002 recovered within 4 days of admission and was discharged from the hospital on day 4 whereas DD001 remained in the ICU for almost 30 days and experienced renal and pulmonary dysfunction. This experiment tested the effect of the serum on the endothelial cell viability. The Molecular Probes Live/Dead Viability/Toxicity Kit was used to assess the health of the cells. The number of live and dead cells was determined using ImageJ image analysis software (NIH). The endothelial cells were treated with 5%, 10%, and 20% of the serum samples added to the complete growth medium for 24- and 48-hour time points.



A



B

Figure 8. Cell viability was analyzed using the Molecular Probes Live/Dead Viability/Toxicity Kit. Endothelial cells were treated with 20% patient serum for 24 and 48 hours. A: The microscopic images are from 48-hour treatments. Green cells are viable cells that have converted calcein AM green with esterase activity. The red cells are non-viable cells that have compromised membranes allowing ethidium homodimer-1 (EthD-1) to enter cells and bind with nucleic acids producing red fluorescence. The images clearly show the disruption of the cellular monolayers when treated with AP serum in comparison to normal serum. 20X magnification. B: The chart compares the different 24- and 48-hour treatments.

The 20% serum treatments had the greatest effect on the viability of the endothelial cells and the results are shown in figures 8 and 9. These results show that the serum does impact the viability of the endothelial cells (normal patients vs acute pancreatitis patients) thus leading to disruption of the endothelium monolayer. This decrease in cell viability is variable within the acute pancreatitis patient population. Based on these findings, similar experiments will be performed on all of the serum samples to determine if the cell viability changes with the day of sample collection at later time points and if there is correlation with patient lab results and vitals.

The propidium iodide and live/dead cell kit assays do not specifically define the mode of cell death. They determine if the cell membrane is damaged which could be indicative of necrotic or apoptotic cell death. In upcoming experiments, methods to define mode of cell death (Aim 2) will be performed. In addition, experiments to determine potential biomarkers (Aim 3) will begin. The serum will be fractionated by gel filtration chromatography on a Superdex 200 (GE Healthcare) column. This chromatography method will allow an increased resolution of the protein separation in the serum samples allowing for more robust cell culture viability experiments using the fractions collected from the chromatography column. A more targeted mass spectrometric analysis will also be possible utilizing these fractions. These techniques will be utilized throughout the remainder of the project.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

Patient recruitment/enrollment into the project will continue. Serum will be tested for effect on endothelial cell viability. In upcoming experiments, methods to define mode of cell death (Aim 2) will be performed. In addition, experiments to determine potential biomarkers (Aim 3) will begin using liquid chromatography and mass spectrometric techniques.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

There was a delay in getting the IRB approval from the University of Pittsburgh IRB Committee as a "less than minimal risk" classification. Approval was received February 4, 2015. This was followed by the US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protections Office (HRPO) approval on February 28, 2015. There was also a delay in hiring a Research Nurse Coordinator for this study. The perfect candidate was hired July 1, 2015.

Changes that had a significant impact on expenditures

There was a delay in hiring a Research Nurse Coordinator for this project. The coordinator was hired July 1, 2015 therefore salary support in year 1 was for 4 months.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications.

Nothing to Report.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

Nothing to Report.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**.

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David C. Whitcomb, MD
Project Role: PI
Nearest person month(s) worked: 2.4 months
Contribution to Project: Dr. Whitcomb oversaw all research in this project. Weekly research meetings were held to disseminate progress. In addition, Dr. Whitcomb wrote the IRB application and interviewed candidates for the Nurse Research Coordinator position.

Name: David G. Binion, MD
Project Role: Co-Investigator
Nearest person month(s) worked: 1.2 months
Contribution to Project: Dr. Binion provided assistance with experiments in this project and participates in research meetings. In addition, Dr. Whitcomb wrote the IRB application and interviewed candidates for the Nurse Research Coordinator position.

Name: Annette S. Wilson, PhD
Project Role: Laboratory Manager
Nearest person month(s) worked: 8.4 months
Contribution to Project: Dr. Wilson coordinated the experiments and performed imaging and data analysis. She participates in the weekly research meetings. In addition, Dr. Wilson assisted Sr. Whitcomb with writing the IRB application.

Name: Weiping DeBlasio, RN
Project Role: Research Nurse Coordinator
Nearest person month(s) worked: 1 month
Contribution to Project: Mrs. DeBlasio has consented all patients currently in the study. She has transported the blood samples to the research lab and assisted in processing, aliquotting, and storing samples. She attends the weekly research meetings. In addition, Mrs. DeBlasio coordinated renewal of the IRB proposal for this study.

Name: William M. Rivers
Project Role: Research Technician
Nearest person month(s) worked: 6 months
Contribution to Project Mr. Rivers was responsible for endothelial cell isolation, cell maintenance, and set up of experiments.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

*LIVE/DEAD® Viability/Cytotoxicity Kit *for mammalian cells**

Quick Facts

Storage upon receipt:

- ≤–20ºC
- Protect from light

Ex/Em

- Calcein = 494/517 nm
- Ethidium homodimer-1 in the presence of DNA = 528/617 nm

Note: Calcein AM may hydrolyze if exposed to moisture.

Introduction

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability—intracellular esterase activity and plasma membrane integrity. Molecular Probes has found that calcein AM and ethidium homodimer (EthD-1) are optimal dyes for this application.[1-3] The kit is suitable for use with fluorescence microscopes or fluorescence multiwell plate scanners and easily adaptable for use with flow cytometers and other fluorescence detection systems. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells[4] and certain tissues,[5,6] but not to bacteria or yeast.[3] This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion, ^{51}Cr release and similar methods for determining cell viability and cytotoxicity. It is generally faster, less expensive, safer and a more sensitive indicator of cytotoxic events than alternative methods. Validity of the LIVE/DEAD® Viability/Cytotoxicity assay for animal cell applications has been established by several laboratories. Published applications include measuring the cytotoxic effects of tumor necrosis factor (TNF),[7] β-amyloid protein,[8] adenovirus E1A proteins,[9] tetrodotoxin (TTX) binding to Na^+ channels,[10] methamphetamines[11] and mitogenic sphingolipids.[12] The assay has also been utilized to quantitate apoptotic cell death[13,14] and cell-mediated cytotoxicity.[15,16]

Principle of the Method

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells.

Kit Contents

- **Calcein AM** (Component A), two vials, 40 µL each, 4 mM in anhydrous DMSO
- **Ethidium homodimer-1** (Component B), two vials, 200 µL each, 2 mM in DMSO/H_2O 1:4 (v/v)

At the recommended reagent concentrations and volumes, this kit contains sufficient material to perform approximately 1,000 tests using a fluorescence microscope or fluorescence microplate reader or approximately 100 tests using a flow cytometer.

Storage and Handling of Reagents

Reagents in this kit should be stored sealed, desiccated, protected from light and frozen at ≤–20˚C. Allow the reagents to warm to room temperature and centrifuge briefly before opening. Before refreezing, seal all stock solutions tightly. Calcein AM is susceptible to hydrolysis when exposed to moisture. Prepare aqueous working solutions containing calcein AM immediately prior to use, and use within one day. EthD-1 is stable and insensitive to moisture. Stock solutions of EthD-1 in DMSO/ H_2O or other aqueous media can be stored frozen at ≤–20°C for at least one year.

Fluorescence Microscopy Protocol

Select the Optical Filters

Calcein and EthD-1 can be viewed simultaneously with a conventional fluorescein longpass filter. The fluorescence from these dyes may also be observed separately; calcein can be viewed with a standard fluorescein bandpass filter and EthD-1 can be viewed with filters for propidium iodide or Texas Red® dye. Typical characteristics of some appropriate filters are summarized in Table 1.

Prepare the Cells

1.1 Adherent cells may be cultured on sterile glass coverslips as either confluent or subconfluent monolayers (e.g., fibroblasts are typically grown on the coverslip for 2–3 days until acceptable cell densities are obtained). The cells may be cultured inside 35 mm disposable petri dishes or other suitable containers; non-adherent cells may also be used.

1.2 Wash the cells prior to the assay to remove or dilute serum esterase activity generally present in serum-supplemented growth media (serum esterases could cause some increase in extracellular fluorescence by hydrolyzing calcein AM). Wash adherent cells gently with 500–1,000 volumes of Dulbecco's phosphate-buffered saline (D-PBS) (note A).

1.3 Wash non-adherent cells in a test tube with 500–1,000 volumes of tissue culture–grade D-PBS and sediment by centrifugation. Transfer an aliquot of the cell suspension to a coverslip. Allow cells to settle to the surface of the glass coverslip at 37°C in a covered 35 mm petri dish.

1.4 Treat the cells with cytotoxic agents as required at any time prior to or concurrent with LIVE/DEAD® reagent staining.

Determine the Optimal Dye Concentrations

Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with calcein AM and of dead cells with EthD-1. The optimal concentrations are likely to vary depending on the cell type. In general it is best to use the lowest dye concentration that gives sufficient signal. The following method can be used to determine optimal dye concentrations:

2.1 Remove the LIVE/DEAD® assay reagents from the freezer and allow them to warm to room temperature.

Table 1. Characteristics of common filters suitable for use with the LIVE/DEAD® Viability Kit

Omega Filters*	Chroma Filters*	Notes
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of calcein and EthD-1 stains
XF22, XF23	31001, 41001	Bandpass filters for viewing calcein alone
XF32, XF43, XF102, XF108	31002, 31004, 41002, 41004	Bandpass filters for viewing EthD-1 alone

* Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

2.2 Prepare some samples of live cells as well as of dead cells on glass coverslips. Kill the cells using any preferred method (e.g., treatment with 0.1% saponin for 10 minutes, 0.1–0.5% digitonin for 10 minutes, 70% methanol for 30 minutes or complement and the appropriate IgG for 30 minutes).

2.3 Using samples of dead cells, select an EthD-1 concentration that stains the dead cell nuclei bright red without staining the cytoplasm significantly (try from 0.1 to 10 µM EthD-1).

2.4 Using samples of dead cells, select a calcein AM concentration that does not give significant fluorescence in the dead cell cytoplasm (try from 0.1 to 10 µM calcein AM).

2.5 Using samples of live cells, check to see that the calcein AM concentration selected in step 2.4 generates sufficient fluorescence signal in live cells (if not, try a higher concentration).

2.6 The reagent concentrations determined in steps 2.3 and 2.5 are optimal for the viability experiments.

Example Dilution Protocol

This example protocol makes 10 mL of an approximately 2 µM calcein AM and 4 µM EthD-1 solution. We found these dye concentrations to be suitable for NIH 3T3, PtK2 and MDCK cells when incubated at room temperature for 20–40 minutes. Cultured mouse leukocytes (J774A.1), which have higher esterase activity, require 5–10 times less calcein AM than that required for the three other cell types, but the same amount of EthD-1. This is an example protocol only; the optimal dye concentrations for any experiment will vary.

3.1 Remove theLIVE/DEAD® reagent stock solutions from the freezer and allow them to warm to room temperature.

3.2 Add 20 µL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture–grade D-PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-1 solution.

3.3 Combine the reagents by transferring 5 µL of the supplied 4 mM calcein AM stock solution (Component A) to the 10 mL EthD-1 solution. Vortex the resulting solution to ensure thorough mixing.

3.4 The resulting approximately 2 µM calcein AM and 4 µM EthD-1 working solution is then added directly to cells. The final concentration of DMSO is ≤ 0.1%, a level generally innocuous to most cells.

3.5 Note that aqueous solutions of calcein AM are susceptible to hydrolysis (see Storage and Handling of Reagents). Aqueous working solutions should therefore be used within one day.

Perform the Viability Assay

4.1 Add 100–150 µL of the combined LIVE/DEAD® assay reagents, using optimized concentrations, to the surface of a 22 mm square coverslip, so that all cells are covered with solution. Incubations should be performed in a covered dish (e.g., 35 mm disposable petri dish) to prevent contamination or drying of the samples.

4.2 Incubate the cells for 30–45 minutes at room temperature. A shorter incubation time may be used if the dye concentrations or incubation temperature are increased.

4.3 Following incubation, add about 10 µL of the fresh LIVE/DEAD® reagent solution or D-PBS to a clean microscope slide.

4.4 Using fine-tipped forceps, carefully (but quickly) invert and mount the wet coverslip on the microscope slide. To prevent evaporation, seal the coverslip to the glass slide (e.g., with clear fingernail polish). Avoid damaging or shearing cells in the preparation of the slides.

4.5 View the labeled cells under the fluorescence microscope.

Fluorescence Microplate Protocol

Select the Optical Filters for the Microplate Reader

In order to obtain the greatest sensitivity using a plate reader, we recommend exciting the fluorophores using optical filters optimal for their respective absorbances. Calcein is well excited using a fluorescein optical filter (485 ± 10 nm) whereas EthD-1 is compatible with a typical rhodamine optical filter (530 ± 12.5 nm). The fluorescence emissions should be acquired separately as well, calcein at 530 ± 12.5 nm, and EthD-1 at 645 ± 20 nm.

Prepare the Cells for the Microplate Reader

5.1 Culture adherent cells in the multiwell plate. Fibroblast cells are typically grown in the wells for 2–3 days until acceptable cell densities are obtained. Wash the cells gently with 500–1000 volumes of Dulbecco's phosphate-buffered saline (D-PBS) prior to the assay (note A). After the last wash, add sufficient D-PBS to at least cover the bottom of the well. The cell samples are washed to remove or to dilute esterase activity generally present in serum-supplemented growth media that could cause an increase in extracellular fluorescence due to hydrolysis of calcein AM.

5.2 Wash relatively nonadherent cells (e.g., leukocytes or other suspended cells) in a test tube with 500–1000 volumes of tissue culture–grade D-PBS and sediment by centrifugation to remove serum esterase activity.

5.3 Add the cells in a sufficient volume of buffer to at least cover the bottom of the wells. In general, for flat-bottomed wells where the total capacity is 250–300 µL, add about 100 µL; for round-bottomed wells where the total capacity is 150–200 µL, add about 70 µL; for conical wells where the total capacity is 100–150 µL, add about 50 µL. Small buffer volumes may be preferred to minimize dilution of cytotoxic agents and other reagents.

5.4 Treat the cells with cytotoxic agents as required at any time prior to or concurrent with LIVE/DEAD® reagent staining.

5.5 The minimum detectable number of cells per well is usually between 200 and 500. The maximum usable number of cells per well is on the order of 10^6.

Determine the Optimal Dye Concentrations

Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with calcein AM and dead cells with EthD-1. Changes in optical filters, instrument sensitivity settings and numbers or types of cells may require different dye concentrations. In general, it is best to use the lowest dye concentration that gives sufficient signal. The following method can be used to determine optimal dye concentrations:

6.1 Remove the LIVE/DEAD® reagents from the freezer and allow them to warm to room temperature. Select appropriate filters and settings on the plate reader.

6.2 Prepare samples of live cells as well as of dead cells. Kill the cells using any preferred method (e.g., treatment with 0.1% saponin for about 10 minutes, 0.1–0.5% digitonin for about 10 minutes, 70% methanol for about 30 minutes or complement and the appropriate IgG for about 30 minutes).

6.3 Using samples of dead cells, determine the saturating concentration of EthD-1 (the lowest concentration that still yields maximal fluorescence). Try from 0.1 to 10 µM of EthD-1, while maintaining a constant high cell concentration (about 10^6 cells per mL). Monitor the time course of staining to determine optimum incubation times (try taking measurements every 10–15 minutes). We found a 45 minute incubation in 4 µM EthD-1 saturates the binding sites in a sample of 120,000 killed mouse leukocytes.

6.4 Using samples of dead cells, determine concentrations of calcein AM that give negligible staining of dead cells (try from 0.1 to 5 µM calcein AM).

6.5 Using samples of live cells, determine the concentration of calcein AM that gives fluorescence in live cells sufficient to permit clear detection. If the signal is too low, increase the number of cells or use a slightly higher concentration of the dye.

6.6 The reagent concentrations determined in steps 6.3 and 6.5 are optimal for the viability assay.

Sample Preparation Example for Microplate Reader Measurements

This example protocol makes 10 mL of the LIVE/DEAD® reagents for use in a multiwell plate scanner at 1 µM calcein AM and 2 µM EthD-1 (we found these reagent concentrations to be optimal for mouse leukocytes). The protocol prepares a 2X concentrated reagent stock to allow for a final two-fold dilution upon addition to the wells. Ten milliliters of the stock solution at 100 µL per test gives enough dye solution for one 96-well microplate. This is an example protocol only; the actual volumes and concentrations used in an experiment will depend on the type of cells and microplates used.

7.1 Remove the LIVE/DEAD® reagent stock solutions from freezer and allow them to warm to room temperature.

7.2 Add 20 µL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture–grade D-PBS, vortexing to ensure thorough mixing. This gives an approximately 4 µM EthD-1 solution.

7.3 Transfer a 5 µL aliquot of the supplied 4 mM calcein AM solution in DMSO (Component A) to the 10 mL of 4 µM EthD-1 solution. Vortex or sonicate the resulting solution to ensure

thorough mixing. This gives an approximately 2 µM calcein AM and 4 µM EthD-1 working solution.

7.4 Distribute 100 µL of cell-containing buffer to each well. Add an additional 100 µL of the LIVE/DEAD® working solution, yielding 200 µL per well containing 1 µM calcein AM and 2 µM EthD-1. The final concentration of DMSO is ≤ 0.1%, a level generally innocuous to most cells.

Fluorescence Measurements Using a Microplate Reader

8.1 Prepare the samples of experimental cells (A and B below) and of live and dead cell controls (C through F below).

8.2 The set of control measurements is included to account for sources of background fluorescence, which can then be factored out in subsequent calculations. Treat the experimental and control cell samples identically (i.e., maintain constant cell numbers, reagent concentrations, and incubation times and temperatures). Label the experimental cells with calcein AM and EthD-1. Label the control samples as indicated with either calcein AM or EthD-1. A cell-free control (G and H below) may be included to test for background fluorescence from the cytotoxic agent being tested or from other additives in the medium.

8.3 Add the LIVE/DEAD® reagents to the wells to the optimal final concentrations (described in *Determine the Optimal Dye Concentrations*).

8.4 Incubate the samples for the optimal time interval (described in *Determine the Optimal Dye Concentrations*), e.g., at room temperature for 30–45 minutes.

8.5 Measure the fluorescence in the experimental and control cell samples using the appropriate excitation and emission filters:

A. Fluorescence at 645 nm in the experimental cell sample, labeled with calcein AM and EthD-1 = $\mathbf{F(645)_{sam}}$
B. Fluorescence at 530 nm in the experimental cell sample, labeled with calcein AM and EthD-1 = $\mathbf{F(530)_{sam}}$
C. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only = $\mathbf{F(645)_{max}}$
D. Fluorescence at 645 nm in a sample where all the cells are dead, labeled with calcein AM only = $\mathbf{F(645)_{min}}$
E. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with EthD-1 only = $\mathbf{F(530)_{min}}$
F. Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only = $\mathbf{F(530)_{max}}$
G. Fluorescence at 530 nm of the cell-free sample with or without dye added = $\mathbf{F(530)_0}$
H. Fluorescence at 645 nm of a cell-free sample with or without dye added = $\mathbf{F(645)_0}$

Interpretation of the Results

The relative numbers of live and dead cells can be expressed in terms of percentages or as absolute numbers of cells (described in *Determining Absolute Numbers of Live and Dead Cells*) at about 530 nm and limited fluorescence signal at longer wavelengths. Dead cells are characterized by intense fluorescence at >600 nm and little fluorescence around 530 nm. Background fluorescence readings ($F(530)_0$ and $F(645)_0$) may be subtracted from all values of F(530) and F(645) respectively prior to calculation of results.

The percentage of live cells can be calculated from the fluorescence readings defined above as:

$$\%\ \text{Live Cells} = \frac{F(530)_{sam} - F(530)_{min}}{F(530)_{max} - F(530)_{min}} \times 100\%$$

The percentage of dead cells can be calculated from the fluorescence readings defined above as:

$$\%\ \text{Dead Cells} = \frac{F(645)_{sam} - F(645)_{min}}{F(645)_{max} - F(645)_{min}} \times 100\%$$

Determining Absolute Numbers of Live and Dead Cells Using a Microplate Reader

The total number of cells in a sample can be counted by killing all of the cells (see step 6.2), labeling with a saturating concentration of EthD-1 and measuring fluorescence at >600 nm. The fluorescence intensity is then linearly related to the total number of cells present in the sample. This may be done at the end of a set of viability experiments in order to express cell viability in terms of absolute numbers of live and dead cells.

9.1 Perform the cell-viability measurements (described in *Fluorescence Measurements Using Microplate Reader*).

9.2 Kill all of the cells in the samples (e.g., by adding about 0.1% saponin to each well; add 2–5 µL per well from a 5% saponin stock solution in distilled water).

9.3 Mix by shaking the plate; wait 10 minutes (or until the signal equilibrates).

9.4 Read the EthD-1 fluorescence at ~645 nm. The fluorescence intensity is linearly related to the number of cells in the sample. This value can be compared to a standard curve of numbers of dead cells vs fluorescence intensity, generated separately by using a saturating EthD-1 concentration on known numbers of dead cells in a microplate.

Flow Cytometry Protocol: Viability Assay

10.1 Allow all reagents to come to room temperature.

10.2 Make an 80-fold dilution of calcein AM (Component A) in DMSO to make a 50 µM working solution (i.e., add 2 µL of Component A to 158 µL DMSO). The working solution should be used within one day.

10.3 Prepare a 1 mL suspension of cells with 0.1 to 5×10^6 cells/mL for each assay. Cells may be in culture medium or buffer.

10.4 Add 2 µL of 50 µM calcein AM working solution and 4 µL of the 2 mM ethidium homodimer-1 stock to each milliliter of cells. Mix the sample.

10.5 Incubate the cells for 15–20 minutes at room temperature, protected from light.

10.6 As soon as possible after the incubation period (within 1–2 hours), analyze the stained cells by flow cytometry using 488 nm excitation and measuring green fluorescence emission for calcein (i.e., 530/30 bandpass) and red fluorescence emission for ethidium homodimer-1 (i.e., 610/20 bandpass). Gate on cells to exclude debris. Using single color stained cells, perform standard compensation. The population should separate into two groups: live cells will show green fluorescence and dead cells will show red fluorescence (Figure 1).

Flow Cytometry Protocol: Viability Assay with CountBright™ Absolute Counting Beads

Note: The accuracy of cell counts based on CountBright™ absolute counting beads depends on sample handling and the precise delivery of the volume of beads. The CountBright™ absolute counting beads must be mixed well to assure a uniform suspension of microspheres; vortex for 30 seconds immediately before removing an aliquot. Cell suspensions may be diluted, but should be assayed without wash steps.

11.1 Allow all reagents to thaw completely.

11.2 Make an 80-fold dilution of calcein AM (Component A) in DMSO to make a 50 µM working solution (i.e., add 2 µL of Component A to 158 µL DMSO). The working solution should be used within one day.

11.3 Prepare a 1 mL suspension of cells with 0.1 to 5×10^6 cells/mL for each assay. Cells may be in culture medium or buffer.

11.4 Add 2 µL of 50 µM calcein AM solution and 4 µL of the 2 mM ethidium homodimer-1 stock per mL of cells. Mix the sample.

11.5 Incubate the cells for 15–20 minutes at room temperature, protected from light.

11.6 Allow the CountBright™ absolute counting beads to come to room temperature. Gently vortex the microsphere suspension for 30 seconds to completely resuspend.

11.7 Immediately after vortexing the counting bead suspension, add 50 µL of counting beads to each milliliter of sample and vortex.

Note: At this dilution, the small amount of Tween 20 and sodium azide contributed by the CountBright™ absolute counting beads has not been noted to affect cell staining or viability.

11.8 As soon as possible after the incubation period, analyze the sample by flow cytometry using 488 nm excitation and measuring green fluorescence emission for calcein (i.e., 530/30 bandpass) and red fluorescence emission for ethidium homodimer-1 (i.e., 610/20 bandpass). Gate both on cells (to exclude debris) and on counting beads. Set forward scatter threshold low enough to include the microspheres on the forward vs side scatter plot (Figure 2). Using single color stained cells, perform standard compensation. The population should separate into two groups: live cells will show green fluorescence and dead cells will show red fluorescence. The CountBright™ absolute counting beads can be distinguished from cells (Figure 3).

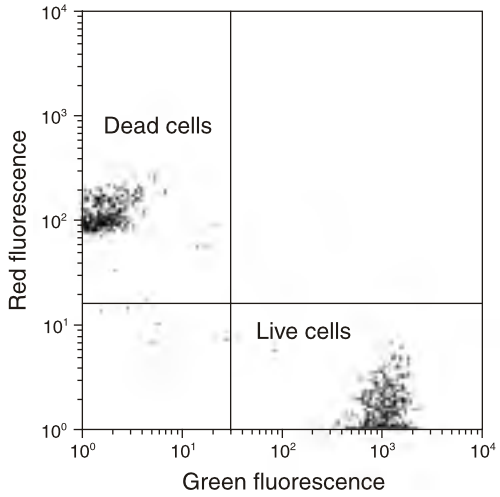


Figure 1. Flow cytometric viability assay using our LIVE/DEAD® Viability/Cytotoxicity Kit. A 1:1 mixture of live and ethanol-fixed human B cells was stained with calcein AM and ethidium homodimer-1 following the protocol provided. Flow cytometry analysis was carried out with excitation at 488 nm. The resulting bivariate frequency distribution shows the clear separation of the green-fluorescent (530 nm) live-cell population from the red-fluorescent (585 nm) dead-cell population.

Note: Collect at least 1,000 bead events to assure a statistically significant determination of sample volume.

11.9 The counting beads should appear in the upper right corner of all fluorescence plots (Figure 3), and can be gated accordingly.

Note: If the CountBright™ absolute counting beads cannot be resolved from cells in a particular emission parameter combination, use a different combination of emission parameters to gate the counting beads.

Calculation of cell concentration:

$$\frac{A}{B} \times \frac{C}{D} = \text{concentration of sample as cells/µL}$$

Where:
A = number of cell events
B = number of bead events
C = assigned bead count of the lot (beads/50 µL)
D = volume of sample (µL)

Example calculation: A 1,000 µL volume of cells was stained. Afterwards, 50 µL of CountBright™ absolute counting beads was added.

$$\frac{1{,}700 \text{ cells}}{1{,}030 \text{ beads}} \times \frac{49{,}500 \text{ beads}/50 \text{ µL}}{1{,}000 \text{ µL}} = 81.7 \text{ cells/µL}$$

Note: The calculation should be corrected if the sample is diluted or if a different volume of CountBright™ absolute counting beads is used.

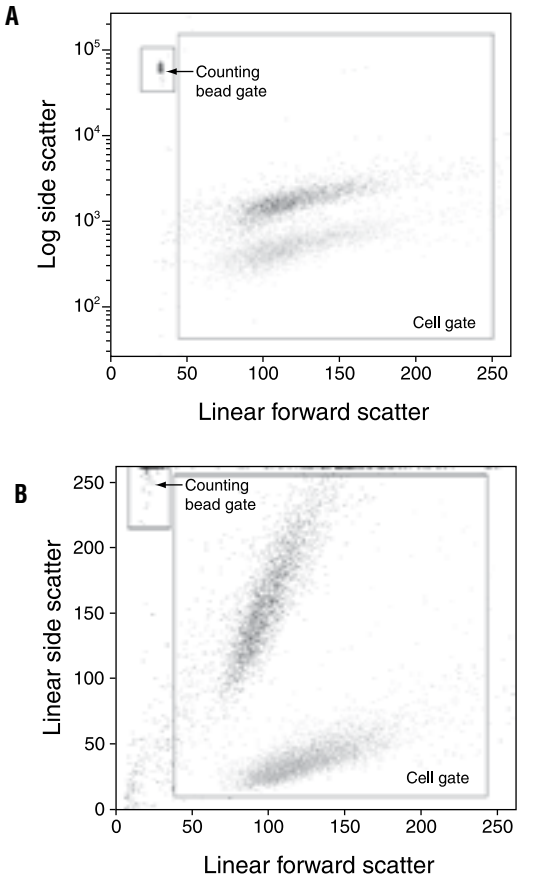


Figure 2. Counting bead gating on forward vs side scatter. A mixture of live and heat-killed Jurkat cells (human T-cell leukemia) was stained with calcein AM and ethidium homodimer-1 following the protocol provided. CountBright™ absolute counting beads were added prior to data acquisition on the flow cytometer. A) Forward scatter vs logarithmic side scatter shows gating of cells to exclude debris as well as gating of counting beads. B) Forward scatter vs linear side scatter shows gating of cells to exclude debris with gating of counting beads. The counting bead gate is adjusted to include the last channel in side scatter.

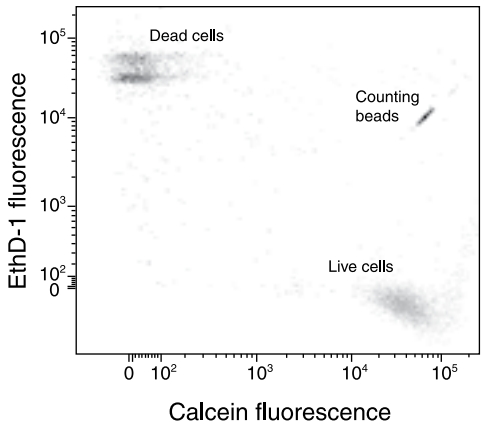


Figure 3. Plot of calcein fluorescence collected through a 530/30 bandpass filter vs ethidium homodimer-1 fluorescence collected through a 610/20 bandpass filter, showing clear separation of live and dead cells, as well as counting beads. A mixture of live and heat-killed Jurkat cells was stained with calcein AM and ethidium homodimer-1 following the protocol provided. CountBright™ absolute counting beads were added prior to data acquisition on the flow cytometer using 488 nm excitation.

Note

[A] Any standard saline buffer may be used throughout these protocols. Colored additives like phenol red should be checked, however, to see if they interfere with the fluorescence (see step 8.2). A suggested buffer is sterile tissue culture–grade D-PBS: KCl (200 mg/L), KH_2PO_4 (200 mg/L), NaCl (8,000 mg/L), and Na_2HPO_4 (1150 mg/L).

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